Human cell-based in vitro systems for vaccine evaluation

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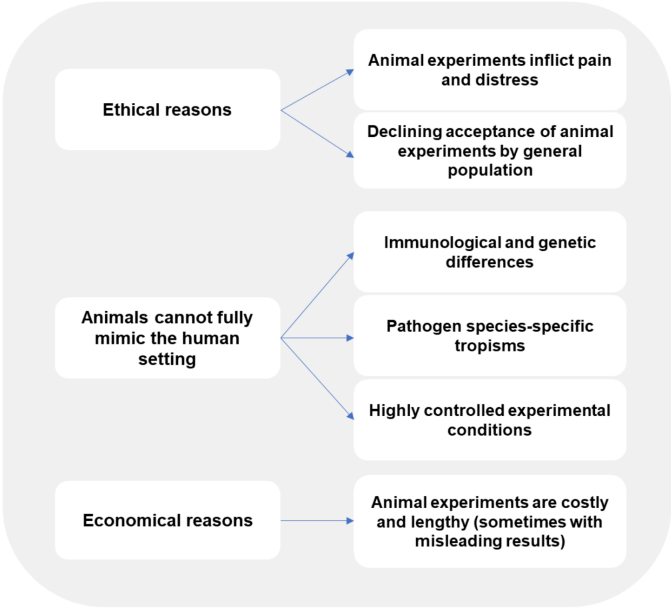
Chapter 6

Discussion

Discussion

Much of the current knowledge we have about vaccines comes from studies in animal models, mainly murine models. While fundamental immunological concepts derived from studies using inbred, knock out, and transgenic mice have been pivotal to extend our understanding of immune responses, there are multiple reasons to favor the development and use of animal-free alternatives (Box 1).



Box 1. Reasons to look for animal-free alternatives

As of 2010, the European Union (EU) adopted a new legislation concerning the "protection of animals used for scientific purposes" (Directive 2010/63/EU). This was mainly based on the 3Rs principle to replace, reduce, and refine the use of animals for scientific research. This legislation also encourages the development and implementation of alternative methods for animal testing [1].

In the context of vaccine development, the use of alternatives to animal models does not mean the complete replacement of animal testing, but rather the inclusion of auxiliary platforms that can aid in the screening of vaccines and vaccine lots. For example, identifying vaccines with low immunogenic potential by employing animal-free alternatives would contribute to the use of fewer animals. Vaccine-related use of animal models is not only restricted to vaccine development but also includes

the monitoring of the production processes of new vaccines and quality control prior to vaccine batch release. This translates into a constant need of animals in order to check the quality of routinely produced vaccine lots (e.g., diphtheria, tetanus, pertussis, Tick-borne encephalitis virus and polio vaccines) [2]. In the context of vaccine development and production, *in vitro* systems can be extremely valuable as they represent an animal-free alternative to assess quality, predict responses and help in elucidating immunological and cellular mechanisms of vaccines and adjuvants.

Given the increased need for alternatives, the ultimate goal of this thesis was to establish a human cell-based system to assess immunological responses to vaccines *in vitro*. We envisioned an approach in which we could dissect different parts of the immune response by focusing on different types of immune cells. Using monocyte-derived dendritic cells (MoDCs) generated from human peripheral mononuclear cells (PBMCs), we first established an *in vitro* platform addressing vaccine-evoked innate immune responses. Subsequently, we developed a platform which allowed us to assess adaptive responses, more specifically T cell-mediated immune responses. We then assessed the performance and suitability of the established platforms by comparing and evaluating immune responses to different types of vaccines. Lastly, we determined how the responses to selected vaccines observed in the human *in vitro* system related to *in vivo* responses as measured in mice. Figure 1 is a graphical summary of the main results obtained from the establishment of our *in vitro* system, also referred as vaccine evaluation system (VES).

Establishing the in vitro system

To develop a vaccine evaluation system (VES), we first focused on innate immune responses using antigen presenting cells (APCs). The innate immune system is the first line of defense against pathogens and its cellular component is composed of different cell types (i.e., DC, monocytes, macrophages and natural killer cells) equipped with receptors to sense "non-self" microbial components [3,4]. These cells do not only play a key role in controlling the spread of pathogens; but more importantly create an optimal environment for the generation of antigen presentation with the upregulation of co-stimulatory molecules (i.e., MHCII, CD80, CD86). Moreover. they secrete cytokines and chemokines (i.e., IFNγ, TNFα, IL-12, IL-4, IL-10, CCL-21, CXCL-12, CCR7), that enable the activation and differentiation of adaptive immune players, T and B cells [5,6]. DCs are known to be professional APCs, hence pivotally connect the innate and adaptive responses and crucially determine the magnitude and type of the adaptive immune responses [7–9].

Previously, the use of APCs from cell lines and primary cells has allowed assessing innate immune responses *in vitro*, enabling to understand and model basic immunological responses e.g., the differentiation process of monocytes into APCs [10,11]. The use of APCs has been studied as an alternative to the use of animal testing for vaccine registration and batch release. These studies have primarily focused on bacterial vaccines (i.e., pertussis and Haemophilus influenzae) and have favored the use of cell lines over primary cells. The authors claimed that the use of cell lines has the advantage of avoiding safety issues due to unknown infectious pathogens potentially present in blood. Another reason they state is the reproducibility, as opposed to the intrinsic donor-to-donor variation found in primary cells [12,13]. Human primary DCs, on the other hand, have been used to study viral vector vaccines [14–17] and adjuvanted vaccines [12,14,18]. These studies, however, have mainly focused on the mechanism of action of the vector or adjuvant and not on the intrinsic characteristics of the vaccines themselves. Hence, we investigated in **Chapter 2** whether MoDCs could serve as a cellular platform to assess and dissect the immunological properties of vaccines *in vitro* by using two commercially available non-adjuvanted vaccines; namely WIV and SU vaccines.

In establishing this platform, our first choice was to use cell lines as opposed to primary cells, since the first ones are fast and easy to use, cell availability is not a concern and reproducibility is expected to be high. However, even the most promising DC-like cell line, MUTZ-3 [19–25], was found to be unsuitable for our purposes. Therefore, we turned to MoDCs generated from human primary cells. Despite the laborious process to isolate and differentiate cells into DCs, human primary cells have the advantage of retaining many characteristics of the cells *in vivo*. And although using primary DCs brings the possible disadvantage of donor to donor variation, it also gives the opportunity to capture human heterogeneity. In testing the MoDCs, we found that in contrast to the MUTZ-3 cell line, these cells were a suitable platform for screening immunogenic properties of vaccine candidates. MoDCs exposed to WIV and SU vaccines readily displayed vaccine-specific response patterns, reflected in different readouts. WIV could successfully stimulate the expression of genes associated with viral immune responses including MYD88, IRF7, and STAT1, upregulate co-stimulatory molecules on the DC surface like MHCII, CD80 and CD86 and induce the production of cytokines, whilst SU displayed a rather poor capacity to induce such innate immune responses. Notably, the effects of WIV and SU on human DCs *in vitro* were in concordance with those previously reported *in vivo* for mouse DCs [26] and from clinical studies [27,28]. Lastly, we showed that freshly isolated and freeze-thawed PBMCs could be used for MoDCs generation and both performed equally well. This indicates that it is possible to cryopreserve

batches of PBMCs for later use and the repetition of tests, thereby greatly increasing the practical feasibility of a MoDC-based vaccine evaluation system.

The established MoDC-based *in vitro* platform can recapitulate vaccine-specific activation patterns upon stimulation. By allowing to capture the human heterogeneity, this platform represents a useful tool to assess vaccine-induced mechanisms and to estimate *in vivo* responses to novel vaccine formulations.

Having established a platform suitable to dissect innate responses to vaccines, the next logical step was to focus on the adaptive immune

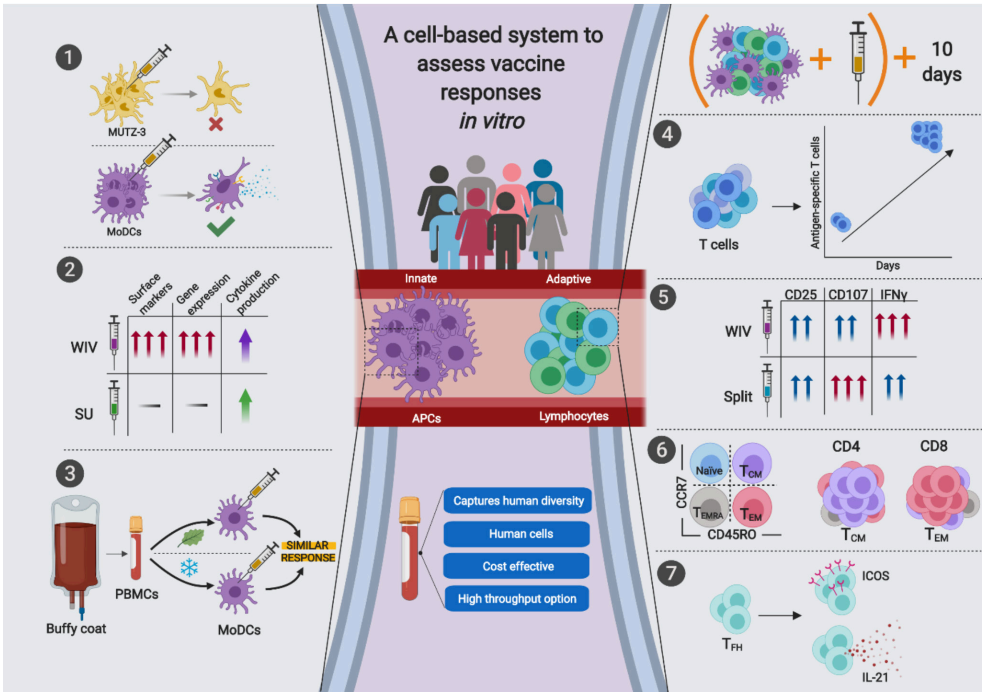


Figure 1. A human cell-based system to assess innate and adaptive immune responses *in vitro***.** Focusing on innate responses, we found that; **1.** Human primary monocyte-derived DCs are a more suitable platform to assess the stimulatory properties of vaccines than the MUTZ-3 cell line; **2.** The use of MoDCs enables the discrimination between high and low immunogenic influenza vaccines (WIV and SU) when measuring different parameters (surface markers, gene expression, and cytokine production); **3.** Freshly isolated and frozen/thawed PBMCs are equally suitable for the generation of MoDCs and respond to vaccines in similar ways. To characterize and assess adaptive responses, we established a system making use of long-term cultures of whole PBMCs. Upon stimulation with different vaccines, this system enables the; **4.** Expansion of vaccine-specific T cell responses over time; **5.** Evaluation of the capacity of vaccines to induce activation of and expression of cytotoxicity markers and IFNγ production by T cells; **6.** Phenotyping of responding T cell subsets (naïve, T_{CM}, T_{EM}, and T_{EMRA}); **7.** Evaluation of the induction of T_{FH} responses. Image created with Biorender.

responses, as these develop subsequently after DC stimulation. Different from innate immune responses, which are quickly generated upon encounter of a pathogen but are unspecific [29], adaptive responses develop slowly, yet they are specific and build-up immunological memory [30,31]. The ultimate goal of vaccination is the generation of antigen-specific (memory) immune responses. To achieve this, vaccines mimic the events that take place during infection. Adaptive responses are divided into T cell-mediated and antibody responses [32,33]. For many infectious diseases (i.e., HIV, malaria, tuberculosis, hepatitis and influenza), T cell-mediated responses have shown to be an important requirement for optimal immune protection [34–42]. A T cell-inducing vaccine should ideally be able (among others) to activate and generate cells than can recognize and kill infected cells. However, vaccines should not only induce effector T cells like cytotoxic T lymphocytes (CTLs) and proper memory T cells; but also, induce proper CD4 responses, by eliciting the right type of T helper response (i.e., T_{H1}, T_{H2}). Additionally, vaccines should also provide enough stimulation for the activation of T_{FH} responses. The magnitude and quality of these T_{FH} responses have a direct effect on the generation and maintenance of germinal centers required for proper B cell activation and thus, the generation of high-affinity antibodies [43–46].

Given these facts, in **Chapter 3**, we developed an *in vitro* platform to assess vaccine-induced T cell responses. Traditionally, T cell responses to vaccines have been studied *in vitro* using DC-T cell co-cultures where DCs are generated from (human) PBMCs, pulsed with antigen and later brought in contact with autologous T cells [47–51]. This is, however, a rather tedious and time-consuming procedure. We therefore studied, whether vaccine effects on T cells could also be measured in long-term cultures of unfractionated PBMCs. Next to being simple and straightforward, this approach enables the cross-talk of multiple immune cell types, which might be beneficial for optimal T cell responses.

Using this *in vitro* platform, we observed that influenza antigen-specific T cells present in human PBMCs, when stimulated with suitable vaccines, expanded over time, adopted an activated phenotype and started to produce IFNγ and the cytotoxicity marker CD107. Given the fact that our blood donors had most likely been exposed to influenza before we assumed that the responding cell population would have been memory T cells. Indeed, characterization of the responding T cells revealed that they almost exclusively expressed the memory marker CD45RO with the majority of CD4 T cells being of the effector memory phenotype and the majority of the CD8 T cells being of the central memory phenotype. This result is well in line with responses to influenza vaccines observed *in vivo*

[52–57]. Remarkably, we also detected a response of circulating T_{FH} cells to vaccines in terms of an increase in the frequency of $ICOS^+CD4^+CXCR5^+$ cells and induction of IL-21 production in this cell population. The number and activation status of circulating T_{FH} cells early after vaccination is known to be indicative of later antibody responses [43–46] and the possibility to study vaccine effects on this cell population *in vitro* is thus highly relevant. Using WIV and split influenza vaccine, we observed that the T cells in our *in vitro* system responded in distinct ways to different vaccine formulations bearing the same antigens. Both vaccines were equally potent in activating $CD4^+$ and $CD8^+$ T cells and T_{FH} cells and in inducing IFNγ production in $CD4^+$ T cells. However, WIV was superior to split vaccine in activating IFNγ production in $CD8^+$ T cells, especially in T_{CM} and T_{EM}. This was well in line with our expectations since WIV is known to be a potent activator of DCs as shown in **Chapter 2** and has the capacity to directly deliver antigen to the cytoplasm from where it can fuel into the MHC class I presentation pathway [58].

Thus, the developed *in vitro* platform for the assessment of T cell responses to vaccines can provide detailed information about vaccine effects on various T cell populations, including T_{FH} cells, which are decisive for antibody responses. As such, the platform can be a valuable tool for assessing vaccine mechanisms *in vitro* and for selecting promising vaccine candidates, at least if the vaccines contain antigens to which blood donors likely have memory T cell responses.

Applying and validating the in vitro system

As reported from preclinical and clinical trials, influenza vaccines derived from different virus subtypes differ in immunogenicity [23,42–45]. Virus strain-related differences in immunogenicity have important consequences for vaccine formulations as they might necessitate adjusting the amount of antigen or the addition of an adjuvant to achieve adequate protection [59–65]. Previous work, reports intrinsic features of the respective vaccines to account for the observed strain-specific differences in immunogenicity [66]. These studies however, did not employ vaccines which had been produced in a consistent way; hence, an adequate comparison could not be performed and a proper elucidation of the immunological differences between influenza virus subtypes remained to be performed. In **Chapter 4**, we set out to exploit the potential of our previously established *in vitro* system to evaluate and compare vaccines derived from different influenza virus subtypes.

For this purpose, we performed a head-to-head comparison of WIV and SU vaccines derived from H1N1pdm09, H3N2, H5N1 and H7N9 influenza virus

subtypes produced under standardized conditions. Using a systematic approach, we first focused on the physicochemical characteristics of the vaccines; subsequently, we evaluated their immunological properties using *in vitro* and *in vivo* approaches. Concerning WIV, our comparisons showed clear-cut physicochemical and immunological differences that enabled the discrimination of high (H5N1), intermediate (H1N1pdm09, H3N2) and low (H7N9) immunogenic influenza virus subtypes. Yet, differences concerning SU vaccines were less noticeable. By comparing the immunological effects of the different virus subtype vaccines *in vitro* and *in vivo*, we further assessed in how far the established *in vitro* platforms (**Chapter 2 and 3**) could recapitulate the *in vivo* responses. Notably, results of both *in vitro* and *in vivo* readouts correlated well.

In conclusion, through this head-to-head comparison, we gained valuable insights into the intrinsic differences between vaccines derived from different influenza virus subtypes. This comparison will help improve poorly immunogenic influenza vaccines as future experiments can now focus on the use of alternative vaccine modalities, e.g. adjuvants that can improve immunogenicity. We additionally corroborated the suitability of our *in vitro* system as promising animal free-alternatives for vaccine screening and evaluation.

Lastly, **Chapter 5** describes the use of the T cell *in vitro* platform to assess whether vaccine immunogenicity can be improved by using particulate delivery systems and manipulating the particle size. As shown by others [67,68] and confirmed by us in **Chapter 2**, subunit vaccines, consisting of soluble proteins, are poorly immunogenic. The use of adjuvants can improve immunogenicity. Adjuvants are classified into immunopotentiators and particulate delivery systems; both assist in inducing and modifying immune responses [69–71]. Particulate antigen delivery systems consist of particles ranging from nano- to micrometer size that can accomplish the delivery and presentation of bound or encapsulated antigens to APCs [72]. Interestingly, different studies have shown the possibility to steer the type of immune response (T_{H1}, T_{H2}) by manipulating the size, shape or rigidity of particulate delivery systems [73–76]. Here, we investigated in how far manipulation of size affects antigen uptake by human moDCs and stimulation of human T cells, the latter using the previously established T cell platform.

For this purpose, we used two different vaccines, influenza SU and Hepatitis B (HBsAg), to assess the effect of coupling vaccines to nano- or micro-polystyrene particles (0,5 and 3 µm) on the immune response. Microscopical observations depicted that nano- and microparticle-coupled vaccines, as well as unconjugated vaccines, could be taken up by DCs

with an apparent similar efficiency. Nevertheless, employing our *in vitro* T cell evaluation platform, we observed that influenza vaccine coupled to 3 µm particles was somewhat more immunogenic than unconjugated SU influenza vaccine. This was reflected in an increased number of $CD8^+$ T cells producing cytokines and in the total amount of cytokines produced. In contrast, coupling of HBsAg to beads did not affect the magnitude of the T cell responses, except that the frequencies of IFNγ- and IL10-producing T cells were slightly higher when the antigen was coupled to 3 µm beads. All our blood donors most likely had been exposed to influenza several times and thus their PBMCs contained memory T cells which could rapidly respond to encounter of influenza vaccine *in vitro*. In contrast, the donors were most likely naïve for Hepatitis B. Nevertheless, we could still detect T cell responses to HBsAg in some of our donors, albeit at low frequency. This indicates the versatility of the developed *in vitro* platform to assess not only vaccine effects on memory T cells but also the capacity of vaccines to activate naïve T cells. Yet, the experimental setup did not allow us to discriminate whether the observed responses were mediated by naïve or memory T cells. Thus, further experiments are still needed to corroborate the induction of naïve antigen-specific T cell responses upon *in vitro* stimulation with HBsAg and other de novo antigens.

Taken together, size modification of influenza and Hepatitis B vaccines displayed a measurable but rather small effect on the induction of T cell responses. Although we did find a significant effect of coupling of influenza SU to 3 µm beads on the amounts of certain cytokines produced by the stimulated T cells, the biological relevance of this is questionable since the magnitude of the effect was small. Our results might also indicate that particle size is of minor importance for downstream immune responses in human PBMCs.

Gaps, challenges and opportunities

Gaps

In the context of research and development of vaccines, there are three key needs; 1) antigen selection and vaccine design; 2) novel technologies and routes of administrations; 3) clinical studies and data interpretation [77]. In this context, the use and further development of the *in vitro* vaccine evaluation platform presented in this thesis would be an exciting approach to help solving one of these needs: antigen selection and vaccine design.

Unraveling the mechanisms of infection and host-pathogen interactions is paramount for a rational vaccine design. Many infectious diseases for which we do not have efficient vaccines yet, display very complex dynamics that hamper the design of long-term protective vaccines. Examples of this

include pathogens like influenza, dengue, tuberculosis and malaria that exhibit complex pathogenesis, wide-ranging variability and have evolved different strategies to evade the immune system [78–80]. Finding a vaccine for these pathogens would require to test a significant pool of antigen targets in order to find protective responses. Hence, a rational vaccine approach should be based on a clear understanding of the immunogenicity of potential key antigens and their interaction with the host. Recognizing the human innate and adaptive responses to specific antigens is thus key to select and design effective vaccines.

In this context, VES, which focusses on innate (DCs) and adaptive (T cells) responses could potentially serve three purposes;

Select vaccines
By comparing different vaccine formulations using cells of the same donors, VES allows evaluating whether one vaccine formulation is better than another one. It allows the comparison of vaccines more easily than in animal experiments and clinical trials. Furthermore, it permits the evaluation of vaccine candidates in donors from different contexts (i.e., sex, age, ethnicity, health status). For example, it would facilitate testing different vaccine formulations in particular target groups, e.g. the elderly, which would be challenging to do by means of clinical trials.

Reveal vaccine mechanisms and ways to achieve the needed protective responses
Using VES we can address the characteristics and functions of potent immunogens and evaluate approaches to steer towards specific immune responses. On the one hand, we can reveal the mechanistic nature of the protective immune response by dissecting innate immune pathways (including involved PRR, surface markers, cytokine, chemokines) activated and/or induced by different types of vaccines. Moreover, we can evaluate the possibility of steering the phenotype (i.e., T_{H1}, T_{H2}) of an existing immune response to favor efficient protection. For instance, by assessing earlier identified pathways related to the activation of the innate immune system in PBMCs of young vs. elderly individuals, we could reveal mechanistic differences between the cell patterns of these two target groups.

Assess vaccine quality
For many vaccines, the use of animal models to verify vaccine batch quality is still mandatory. Surprisingly, the number of animals required for these purposes outnumbers that used for scientific research [81]. Currently, there are several initiatives to make such experiments

oblivious by following a 'consistency approach' [82–84], this means the "in-process" monitoring during vaccine production rather than focusing on final batch testing. This monitoring relies solely on *in vitro* biochemical, physicochemical and cellular tests to guarantee the consistency of the new batch with previous ones [83–85]. An *in vitro* approach, as described in this thesis, would allow determining the capacities of vaccine batches to stimulate APCs or T cells and would as such contribute to a reduction of animal use.

Challenges

It is clear that our *in vitro* platform is still a reductionist approach to capture the complexity of the human immune system; and that it cannot fully recapitulate complex interactions. Essential components that can affect the immune response might not be captured (i.e., lymph nodes, germinal centers, gut microbiota). Furthermore, additional validation studies need to be performed to determine in how far the *in vitro* results reflect *in vivo* responses and to what degree they are predictive for vaccination outcome. For instance, in our *in vitro* studies we have shown that WIV was superior to SU vaccine in stimulating APCs and T cells and these results are well in line with *in vivo* results from previous preclinical and clinical studies [27,59,86,87]. However, what is still missing is a head-to-head comparison of immune responses *in vitro*, *ex vivo* and *in vivo* to a specific vaccine. For this, *in vitro* effects of vaccines on PBMCs from unvaccinated individuals should be compared to effects of the vaccine on PBMCs measured *ex vivo* shortly after vaccination and be related to the final vaccination outcome in terms of antibody titers and/or number of T cells induced. With this, we would have proof that a vaccine that performs well *in vitro* does the same *in vivo*. Additionally, it would be required to define a response profile having predictive value for the *in vivo* situation. For this purpose, we could potentially make use of the biomarkers recently discovered by systems vaccinology [88].

Opportunities

The recent boom of systems vaccinology has expanded our knowledge on the molecular basis of the response to conventional commercial vaccines for influenza [89–92] and yellow fever [93,94] and vaccines under development against HIV, dengue and Ebola [95–97]. By using high-throughput 'omics' approaches on PBMCs from vaccinated individuals, systems vaccinology has identified key biomarkers and responses predictive of vaccine-induced protection. It is now clear that PBMCs are an important source of information; and that early immune signatures can be used to predict later responses (i.e., protective levels of antibodies) [89–94]. The most relevant examples are the identification of GCN2 and CaMKIV as predictors of vaccine responses.

Expression levels of GCN2 in PBMCs from recently vaccinated individuals were shown to be predictive of CD8 T cells responses and neutralizing antibody titers against yellow fever vaccines [93,94,98]. CaMKIV expression levels have been shown to inversely correlate with antibody responses in individuals vaccinated with influenza trivalent inactivated vaccines (TIV) and could reliably predict vaccination outcome as early as three days after vaccination [99]. More recently, the generation and activation of circulating T_{FH} (identified as $CD4^+$, $CXCR5^+$, $ICOS^+$, $IL\text{-}21^+$) cells shortly after vaccination were found to correlate with the quality and magnitude of the resulting antibody responses in the context of influenza [46,100–102] and HIV vaccines [103,104].

High throughput "omics" approaches are hypothesis-generating; this means, they perform analysis based on significant amounts of acquired data to delineate hypotheses. However, these hypotheses need to be confirmed, dissected and exploited in conventional assays like the *in vitro* system we have developed. For example, evaluating previously identified biomarkers *in vitro* could also help us in understanding their mechanisms of action by correlating them to the generation of new specific cells subsets, their frequency (percentages), activation state and function (cytokines and proliferation potential).

An additional opportunity for VES is to test vaccine effectiveness on an individual level. Vaccine failure and/or vaccine effectiveness can be influenced by many characteristics of the recipient such as age, vaccine history, health status, ethnicity, sex, or pregnancy [105–107]. For instance, infants (below 1 year) do not respond appropriately to meningococcal vaccine; hence, multiple doses are required [108,109]. Also, it is known that with age, the immune system loses its capacity to respond to infections and vaccinations adequately [110]. VES could be employed using PBMC donors from infants or the elderly to evaluate the reasons behind this and to find alternatives (adjuvants) to improve vaccination outcome.

As of today, we have 26 vaccines against different types of human infectious diseases, and 24 additional vaccines are under development (Table 1) [111]. Available vaccines could allow us to further explore our VES, i.e., by checking whether previously described biomarkers (i.e., GCN2 and CaMKIV) also show up in the developed *in vitro* system. Assessing the available vaccines would also be an excellent chance to gain more knowledge into successful mechanisms of protection. Having a clear understanding of how these vaccines work can provide insights into the development of better candidates or the improvement of others. Knowing that these biomarkers correlate well in our system, we could 1) better understand and dissect the induction of vaccine-related responses, 2) check the induction of responses in individuals with different age, sex and geographic context

Table 1. Vaccines available and in the pipeline.

Available Vaccines		
Cholera	Haemophilus influenzae type b	Meningococcal meningitis
Dengue	Human papillomavirus (HPV)	Malaria
Diphteria	Influenza	Measles
Hepatitis (A, B, E)	Japanese encephalitis	Mumps
Pertussis	Pneumococcal disease	Poliomyelitis
Rabies	Rotavirus	Rubella
Tetanus	Tick-borne encephalitis	Tuberculosis
Typhoid	Varicella	Yellow fever

Pipeline Vaccines		
Campylovacter jejuni	HIV-1	Respiratory Syncytial Virus
Chagas disease	Human Hookworm Disease	Schistosomiasis Disease
Chikungunya	Leishmaniasis Disease	Shigella
Dengue	Malaria	Staphylococcus aureus
Enterotoxigenic E. coli	Nipah virus	Streptococcus pneumoniae
Enterovirus 71	Nontyphoidal Salmonella Disease	Streptococcus pyrogenes
Group B Streptococcus	Norovirus	Tuberculosis
Herpes Simplex Virus	Paratyphoid fever	Universal Influenza Vaccine

and 3) compare between vaccines, if multiple candidates are available for the same pathogen.

Future Perspectives

Comparing vaccine-related responses in young and old individuals

Vaccines still perform suboptimal in the elderly because general vaccine design and formulation is not aimed at the elderly and the molecular mechanisms behind decreased vaccine responses in elderly remain incompletely understood.

Poor vaccine responses are mainly due to immunosenescence, which describes the fact that all of the components of the immune system suffer from aging and progressive loss of responsiveness [112,113]. This process is not only reflected in a restricted ability of the aging immune system to protect the body against pathogens but also in a limited response to vaccination [102,114]. It is known that aging is associated with alterations in the differentiation potential towards memory subsets of T and B cells [56,115]. The number of naïve T cells decreases together with the TCR repertoire, constriction of this repertoire contributes to a failure to respond to different pathogens [116]. Regrettably, innate immune cell types also display age-related alternations [117]. For instance, the function of neutrophils, granulocytes and natural killer (NK) cells is impaired in older adults [117]. In

addition, in macrophages and DCs, Toll-like receptor (TLR) dysregulation together with lower expression of co-stimulatory molecules have also been demonstrated [118–120].

Due to an impaired immune system, the severity of infectious diseases in the elderly is much higher than in younger adults. Moreover, infections are associated with long-term repercussions affecting daily activities, onset of frailty and decrease of independence [121–124]. Thus, prevention of infectious diseases through vaccination is an important challenge to ensure healthy aging. Yet, traditional vaccines against influenza, *Streptococcus pneumoniae*, herpes zoster, tetanus, diphtheria and pertussis have all been found to be less effective in the elderly [125–128]. In the context of influenza vaccines, a meta-analysis showed that vaccine effectiveness in the elderly (65 + population) is 49% for laboratory-confirmed influenza cases [129] compared to 59% in adults between 18-65 years of age [130]; which indicates the need for more potent vaccines [114,130,131].

The established *in vitro* platforms would be ideal to compare vaccine-related immune responses from young and old individuals and thus give an unprecedented chance to better understand why vaccines perform poorly in the elderly; which is not possible using animal models. This comparison could give us insights into; 1) the mechanistical background of immune-related differences between young and old individuals in their response to vaccine and 2) the evaluation of new strategies to ameliorate the responses to vaccines through new adjuvant compounds. As an additional feature this approach would also allow us to better understand variation within heterogeneous populations with distinct genetic and environmental background. In particular, the different exposure history of young and elderly, including the virus strain they encountered first in life, is expected to have effect on the vaccine-induced responses [132]. Overall, this system would allow to compare different vaccines in a much easier and feasible way than testing them in animal models or in clinical trials.

Expanding towards B cell responses

A remaining challenge is the establishment of an *in vitro* system which allows the evaluation of vaccine-induced B cell responses. Typically, vaccines aim at avidly stimulating B cells to induce the production of protective antibodies [133]; however, B cells can also amplify and suppress immune responses by mechanisms different from antibodies [134–137]. Similar to DCs, B cells possess TLRs, present antigens and produce cytokines, thus exerting regulatory and effector functions [134–138]. Given these features, it would be very interesting to design a B cell-based screening system to assess the immunogenicity of vaccines *in vitro*. Such a platform would allow for zooming in into different aspects of the immune response like

the differentiation of different B cell subsets and their proliferation, the induction of cytokine production, and finally the production of antibodies (Table 2). By combining such a B cell system with the previously established platforms, it would be possible to have an integrated and comprehensive view of key immune cell players in the context of vaccine-induced immune responses.

Table 2. Potential processes to study *in vitro* during B cell-induced responses.

Event	Parameter to measure	Technique	Reference
Naïve B cell activation	MHCII, CD86	Immunofluorescence – Flow cytometry	[139]
Memory B cell activation	MHCII, CD86	Immunofluorescence - Flow cytometry	[140–143]
B cell differentiation	AID, Blimp-1, BAFF, Plasma cells, Plasmablasts	qPCR - Flow cytometry	[139,144–146]
Proliferation	CFSE, Ki-67	DNA synthesis/HTS - Flow cytometry	[139,141,147]
Cytokine production	IL-4, IL-6, IL-10, IL-12, IFNγ,TNFα	ELISpot – ELISA	[142,143]
Antibody production	Antigen-specific IgG, IgM	ELISpot – ELISA	[91,148–150]

Building such a system would require first to confirm whether B cells *in vitro* would respond to vaccines at all using the parameters listed in Table 2. Subsequently, the identification of key downstream mechanisms modulated by vaccines, such as subset differentiation and cytokine production, could need to be performed. Few studies have exploited the potential of B cells *in vitro* [139,143,147,151–155], these studies used TLR agonists and not antigens, hence showing the feasibility of a B cell platform with an antigen-independent B cell stimulation approach. Lanzavecchia and colleagues shed light onto important mechanisms of naïve B cell stimulation *in vitro* [139,147,151,152]. Mimicking T cell help with CD40L together with TLR9 agonists they showed the possibility to induce B cell activation and thus proliferation and induction of cytokines [139]. Others demonstrated that also memory B cells can be activated with a combination of CpG and CD40, and can be differentiated into plasmas cells and plasmablasts [143,153]. Jung and colleagues went further into the effects of CpG on the different B cell subtypes *in vitro* concluding that this TLR9 agonist had an effect in the terminal differentiation, indicated by an increase in generation and proliferation of plasma cells from naïve and memory B cells. [154]. As for antibody production, *in vitro* stimulation with a TLR7 ligand induced the secretion of IgM and IgG by naïve B cells [155] and IgM, IgG and IgA by memory B cells [143].

Antigen-dependent B cell activation *in vitro* has remained challenging until recently. This has been mainly due to haplotype variation in MHCIIs requiring specific T cells for B cell activation [156]. In an effort to generate therapeutic antibodies, a recent study showed the possibility to overcome the stringent requirements for antigen-specific stimulation by using streptavidin nanoparticles conjugated to both CpG and antigen [157,158]. Using this approach, the Batistas' lab showed the possibility to identify, quantify and characterize B cells producing antigen specific antibodies. By stimulating memory B cells from healthy donors with CpG in combination with tetanus toxoid or influenza hemagglutinin, they demonstrated the induction of plasma cells able to secrete antigen specific antibodies [158].

In an exploratory approach, we have performed experiments on vaccine-stimulated B cells *in vitro*. Our initial results showed the upregulation of different genes related to B cell fate (encoding for Blimp-1, Pax5 and AID) upon stimulation with WIV but not SU vaccines, as well as the induction of proliferation of WIV-treated but not SU-treated cells as measured by CFSE staining. Yet, according to our experience, although possible, the establishment of an *in vitro* B cell system for vaccine evaluation will be a challenging mission. Different from the T cell approach, cell availability is a problem (there are only around 10% of B cells in PBMCs); next to this, there are no studies so far that assess the effect of mitogens nor antigens in entire B cell populations *in vitro*, rather the available papers start from certain subpopulations like naïve or memory B cells [139,151,158,159]. This could mean that whole PBMCs are not suitable to assess B cell responses and subpopulations would need to be isolated necessitating the use of several antibodies for the isolation of the required populations and further decreasing the number of cells that can be obtained from a given blood sample.

Optimizing this system will require to carefully phenotype and pinpoint those B cell subsets that could give the most relevant information in the context of vaccine induced responses. Moreover, since antigen-activated B cells may undergo different differentiation fates (i.e. memory B cells, short-lived and long-lived plasma cells) and with different requirements for stimulation (with or without T cell help) [101,160,161], it will be necessary to carefully evaluate each of these possibilities to find the best approach to assess B cell vaccine-induced responses *in vitro*.

Concluding remarks

PBMCs can give important information on the immune system and the mechanisms of protection induced by vaccines [89,162–166]. In the context of vaccinated individuals, PBMCs have enabled to pinpoint biomarkers and predictive responses for vaccine protection [43,44,46,102,167–169].

Given this fact, we hypothesized that PBMCs could represent a strong tool in vaccine development. Using influenza as our main model antigen we therefore developed a MoDC-based system to study vaccine effects on APCs and a system using unfractionated PBMCs to study vaccine effects on T cells. We then used the established platforms together with various vaccine formulations to (1) assess vaccine-induced innate immune cell activation of MoDCs; (2) characterize details of vaccine-induced T cell responses; and (3) validated the system against mice *in vivo* responses. In doing so, we found that human primary cells are a better platform to assess responses to vaccines *in vitro* than cell lines and that activation properties of primary MoDCs distinguished different vaccines formulations. After stimulation of whole PBMCs with different vaccine formulations, T lymphocytes displayed distinct magnitudes of response with respect to proliferation and cytokine production. This system also enabled the evaluation of T_{FH} responses, indicating a potential predictive role for antibody responses. In exploiting this platform, we compared vaccines derived from different influenza virus subtypes revealing that different virus subtypes induce different levels of immunogenicity *in vitro* which reflect their immunogenicity in immunized mice.

Our human cell-based platform to assess responses to vaccines *in vitro*, provides an interesting animal free-alternative to circumvent ethical issues and to fulfill the need of representative models of the human situation (given the poor predictive value of animal models [170–172]). The developed platforms intend to be an auxiliary tool for the screening of vaccine candidates. Identifying poorly immunogenic vaccine candidates *in vitro*, would allow to reduce the number of animals needed for preclinical stages decisively. Moreover, it will enable the selection of strong vaccine candidates with better odds to perform well in a clinical setting. Furthermore, the VES can give important insights into the molecular and cellular mechanisms behind vaccines and adjuvants. Seemingly, an extension of this *in vitro* platform could assist in assessing vaccine responses in risk groups (i.e. elderly), ultimately allowing the development of improved vaccines for specific target populations.

In conclusion, this human cell-based platform to assess responses to vaccines *in vitro*, represents a fast and sustainable system with the ability to reflect/recapitulate responses of the human immune system; hence, its use could potentially aid in solving current issues of vaccine development.

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